REMARKS

Upon entry of this amendment, claims 1, 2, 5- 8, 11, 12, 16-19, 22, 25, 26, and 33-41 will be pending. Support for the amendment and new claims can be found throughout the specification and originally filed claims, but in particular at page 5, lines 17-22; page 8, lines 6-8; page 21, line 10 to page 22, line 5; Examples 1and 2 at page 24, line 26 to page 34, line 9; and the figures accompanying Examples 1 and 2. Applicants submit no new matter was added by the amendment or by the addition of new claims.

I. Specification

The specification was objected to because of the reference to patent applications. Specifically, the Examiner points to page 25, line 25. The application cited therein is still pending. Applicants will make the necessary amendments before the issuance of this application as a patent.

II. Claim objections

The Examiner objects to claims 8, 11, 16-17, 19, 22, and 26 as allegedly reciting non-elected species and holds the objection in abeyance until allowable subject matter is identified. As the objection is held in abeyance, Applicants submit that no amendment is necessary at this time.

III. Rejection under 35 U.S.C. §112, first paragraph

Enablement

All previously pending claims were rejected under 35 U.S.C. §112, first paragraph as not fully enabled by the specification. The Examiner alleged that the scope of the claims was not commensurate with the disclosure of the specification. Applicants maintain that the specification adequately supports the complete scope of the claimed invention. Reconsideration of the rejection is respectfully requested in view of the following remarks.

In paragraph 17 of the Office Action, the Examiner asserts that target morphogens need to be identified, in particular to practice claims 6 and 7. Applicants maintain that identifying

which morphogen is exhibiting the observed activity is not necessary to practice the claimed invention, with further remarks as follows:

As described in the specification and in literature, a morphogen triggers a cascade of cellular events, many of which are detectable and/or observable. Each of these observable events is a distinct, specific biochemical change (for example, increase in a certain enzyme activity), and can be considered a result of morphogen activity. These events, being part of a cascade and manifesting differently in different cell types or environment, nevertheless culminate as growth and maintenance of differentiated cells in the particular environment in which these cells are found. Therefore, Applicants maintain that the fact that there is a variety of observable effects of a morphogen, any of which may be measured, does not make the overall effect of morphogen activity on the cells unpredictable or necessitate determination of what activity to measure: a skilled artisan may choose any activity to measure using a convenient assay, such as the assay described in the specification. Any such assay will be useful to measure the reduction of inhibition of a morphogen's overall effect on a cell. To articulate that the invention concerns itself with the overall result of such reduction of inhibition, Applicants amended claim 1 to recite the neuron's proliferation, growth, and maintenance of the differentiated state.

Furthermore, there are means known in the art to identify morphogens if need be: for example, northern blots and immunostains are available to identify expression and phosphorylation status of morphogens, as described in the specification and in documents that are incorporated by reference into the specification. Further, if a morphogen is being added to the composition, Applicants submit that any morphogen described in the specification is appropriate, as it is known in the art that morphogens "crosstalk," *i.e.* one morphogen can replace another in terms of function. See, for example, Sampath *et al., Proc. Natl. Acad. Sci. USA* 1993 Jul 1;90(13):6004-8; Padgett *et al., Proc Natl. Acad. Sci. USA.* 1993 Apr 1;90(7):2905-9 (Exhibits A and B).

Therefore, while the Examiner is correct that, for claims 6 and 7, one who practices the invention necessarily knows which morphogen is being used, any morphogen described in the

specification is sufficient to practice claims 6 and 7 without undue experimentation, as any of them is expected to be useful.

In paragraph 19 of the Office Action, the Examiner states that the claims recite genera of molecules that are defined by their functions. The Examiner asserts that no meaningful search can be carried out based on the functionality of compositions recited in the specification. Applicants submit that, because of the wide variety of compounds useful for this invention, as demonstrated by the examples that Applicants have presented, to fully claim the scope of Applicants' invention, the description is appropriate. Applicants respectfully refer the Examiner to *In re* Swinehart, 439 F.2d 210 (CCPA 1971), that states a functional limitation itself does not render a claim improper. See also MPEP 2173.05(g). Applicants' claims are clearly defined by terms that allow a person practicing a similar method to determine whether or not he or she is within the scope of the claims. Applicants therefore submit that the functional limitation is adequate in the instant application.

Further, Applicants submit that the specification provides sufficient disclosure to support and enable the recitation. Examples of molecules that increase morphogen activity by reducing morphogen inhibition by various factors are found in the specification: namely gp130 as an antagonist of a neuropoietic cytokine, leukemia inhibitory factor (LIF); phosphatidylinositol-specific phospholipase C (PI-PLC) as an antagonist for another neuropoietic cytokine, ciliary neurotrophic factor (CNTF); and (2-*p*-bromocynnamylaminoethyl)-5-isoquinolinesulfonamide, an enantiomer of dibutyryl cAMP, and an enantiomer of cAMP as cyclic-AMP-dependent protein kinase inhibitors. Although they are not within the present claims, Applicants also previously provided inhibitors of the ERK1/ERK2 pathway as examples to support Applicants' contention that the molecules are structurally diverse (Kim et al., Exhibit B of the response to office action, filed January 22, 2004 ("Kim")). These antagonists are characterized as being able to overcome inhibition of morphogen activity *in vitro*.

In paragraph 21 of the Office Action, the Examiner disputes Applicants' remarks that dosage and administration are routinely determined and devised by physicians, and that the information presented in the specification is adequate guidance for one skilled in the art. As a preliminary matter, a copy of Benet *et al.* cited in Applicants' previous response is submitted

herewith. (Exhibit C), to support their contention that the dosage and timing of administration of a pharmaceutical composition must always be individually determined and therefore routine in practice of the relevant art.

In paragraph 23 of the Office Action, the Examiner rejected the claims based on the assertion that the application is not enabling for *in vivo* conditions. While Applicants maintain, as previously presented, that the specification is indeed enabling for the claims, the newly added claims relate to *in vitro* methods and are not objectionable.

Written description

Claims 1, 2, 5-8, 11, 12, 16-19, 22, 25, and 33-37 were rejected under 35 U.S.C. §112, first paragraph, as failing to comply with the written description requirement. Without conceding to the correctness of the Examiner's statement, Applicants have amended the claims to more particularly describe what they consider to be the claimed invention, and also added new claims to clarify the scope of the claimed invention. Reconsideration is respectfully requested.

IV. Rejection under 35 U.S.C. §112, second paragraph

Claims 1, 5, 6, 8, 11, 12, 19, 22, 25, 26, 33-35, 37 and 38 were rejected as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

The Examiner states in paragraph 36 of the Office Action that the term "morphogen activity" is indefinite. As explained above, Applicants submit that "morphogen activity" is not an indefinite term because, although the observable phenomena that are induced by a morphogen can be varied, the overall effect of a morphogen, *i.e.*, the physiologically relevant activity of a morphogen, is to maintain and promote growth of differentiated tissue. Applicants submit that, to one skilled in the art, the term "morphogen activity" has a definite meaning that can be discerned by reading the specification and what was known in the art at the time of filing. Nevertheless, claim 1 was amended to specify what is observed as a result of the morphogen activity in a cell.

In response to paragraphs 40 and 41 of the Office Action, Claim 35 was amended to recite "osteogenic protein 1" for OP-1. Claim 38 was amended to recite "ciliary neurotrophic factor" for CNTF.

V. New Claims

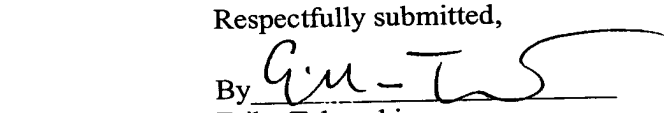
New claims 39-41 were added to distinctly recite aspects of Applicants' claimed invention. Applicants submit that no new matter was added by these claims, and that these claims are in fact a subset of what has already been presented before the Examiner and what has been examined. As stated above, the new claims are submitted in the hopes of advancing prosecution and identifying allowable subject matter. Applicants respectfully request that allowable subject matter be indicated.

In view of the above amendment, Applicants believe the pending application is in condition for allowance. Should the Examiner find the some claims still lacking, Applicants respectfully request that allowable subject matter be indicated to advance the prosecution of this application.

Applicants believe no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 18-1945, under Order No. JJJ-P01-569 from which the undersigned is authorized to draw.

Dated: July 18, 2005

Respectfully submitted,

By 

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Biochemistry

Drosophila transforming growth factor β superfamily proteins induce endochondral bone formation in mammals

(decapentaplegic protein/60A protein/bone morphogenetic proteins/osteogenic protein 1/bone induction)

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Communicated by Elizabeth D. Hay, March 22, 1993

ABSTRACT **Both decapentaplegic (dpp) protein and 60A protein have been implicated in pattern formation during *Drosophila melanogaster* embryogenesis. Within the C-terminal domain, dpp and 60A are similar to human bone morphogenetic protein 2 (75% identity) and human osteogenic protein 1 (70% identity), respectively. Both recombinant human bone morphogenetic protein 2 and recombinant human osteogenic protein 1 have been shown to induce bone formation *in vivo* and to restore large diaphyseal segmental defects in various animal models. We examined whether the *Drosophila* proteins, dpp and 60A, have the capacity to induce bone formation in mammals by using the rat subcutaneous bone induction model. Highly purified recombinant dpp and 60A induced the formation of cartilage, bone, and bone marrow in mammals, as determined by histological observations and by measurements of the specific activity of alkaline phosphatase and calcium content of the implants, thereby demonstrating that related proteins from phylogenetically distant species are capable of inducing bone formation in mammals when placed in sites where progenitor cells are available.**

Embryonic bone development begins with migration of mesenchymal cells to a predetermined site where they either condense, proliferate, and differentiate directly into bone-forming cells or pass through an intermediate cartilage stage before they are replaced with bone. In adult life, bone has a remarkable potential to repair itself upon fracture through a process that recapitulates embryonic bone development. Urist (1) and Reddi and Huggins (2) have shown that the cellular events involved in embryonic bone development are reproduced in predictable intervals in subcutaneous implants of demineralized bone matrix in rats. By employing a reconstitution assay in the rat subcutaneous bone induction model (3, 4) and molecular cloning approaches, several osteogenic proteins (OPs), also called bone morphogenetic proteins [BMPs; BMP-2 through BMP-6, OP-1 (also called BMP-7), and OP-2] have been identified (5–8). The predicted amino acid sequences of these proteins indicate that they are all members of the transforming growth factor β (TGF-β) superfamily, sharing a high degree of homology within the C-terminal seven-cysteine domain (9).

The TGF-β superfamily members are signaling molecules thought to be responsible for specific morphogenic events during development (9, 10). For example, increasing concentrations of *Xenopus* activins can cause animal cap cells to differentiate into various cell types (11) while BMP-4 (closely related to BMP-2) can instruct a ventral posterior positional cell fate on developing mesoderm in the *Xenopus* blastula (12, 13). In the mouse, localized expression of BMPs has been reported in skin, heart, nervous system, and developing limbs (14). A recent study demonstrates that mutation of BMP-5 causes subtle defects in skeletal structures in the mouse (15). In *Drosophila*, the decapentaplegic (dpp) protein specifies dorsal cell fate in the developing embryo and is also involved in the regulation of homeotic gene expression in gut morphogenesis and proximal–distal appendage development in the adult fly (16–19). The developmental function of the *Drosophila* 60A is presently not known, although it is expressed throughout early embryonic development (20, 21).

A comparison of amino acid sequences within the conserved seven-cysteine domain (TGF-β domain) indicates that dpp is more closely related to BMP-2/4 (75% identity), and 60A is more closely related to BMP-5/6 and OP-1 (BMP-7) (70% identity). Both recombinant human BMP-2 and recombinant human OP-1 have been shown individually to induce bone formation in the rat subcutaneous model and to restore large diaphyseal segmental defects in animal models (22–26). The amino acid sequence similarity of dpp and 60A to human BMPs suggests that they might have the ability to induce *in vivo* bone formation in mammals. In the present study, we demonstrate that the recombinant mature disulfide-linked homodimers of dpp and 60A induce the formation and differentiation of endochondral bone in the rat subcutaneous bone induction model.

MATERIALS AND METHODS

Expression and Purification of *Drosophila* dpp and 60A Proteins. *Drosophila* dpp and 60A proteins were produced using the *Drosophila* S2 cell expression system (21, 27). In brief, the full-length cDNA clone encoding either dpp or 60A protein was incorporated into an expression plasmid that contained the metallothionein promotor and leader. The expression plasmid DNA was cotransfected with a selectable dihydrofolate reductase gene. The *dpp* gene product was produced as a processed mature disulfide-linked dimer that was secreted into the medium and, subsequently, one-half of the dpp protein bound to the tissue culture plate. The dpp protein that bound to the plates was extracted with 250 mM $CaCl_2$/0.1% octyl β-glucoside/20 mM Mes, pH 7.2 (plate wash). The 60A protein was also produced as a processed mature disulfide-linked dimer, which was secreted into the medium.

The dpp and 60A proteins were purified from the plate wash and medium, respectively, using two chromatographic steps: S-Sepharose (Pharmacia) and reverse-phase HPLC (C_{18} Vydac). A typical purification utilized 50 ml of plate wash or medium containing 12.5% (vol/vol) fetal calf serum. The plate wash or medium was diluted with 2 vol of 9 M urea/20 mM Mes, pH 6.5, and applied to a 10- to 20-ml S-Sepharose column equilibrated with 6 M urea/20 mM Mes, pH 6.5/50 mM NaCl. After washing with the equilibration buffer, step elution of the bound protein was accomplished

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Abbreviations: BMP, bone morphogenetic protein; OP, osteogenic protein; TGF-β, transforming growth factor β; dpp, decapentaplegic.

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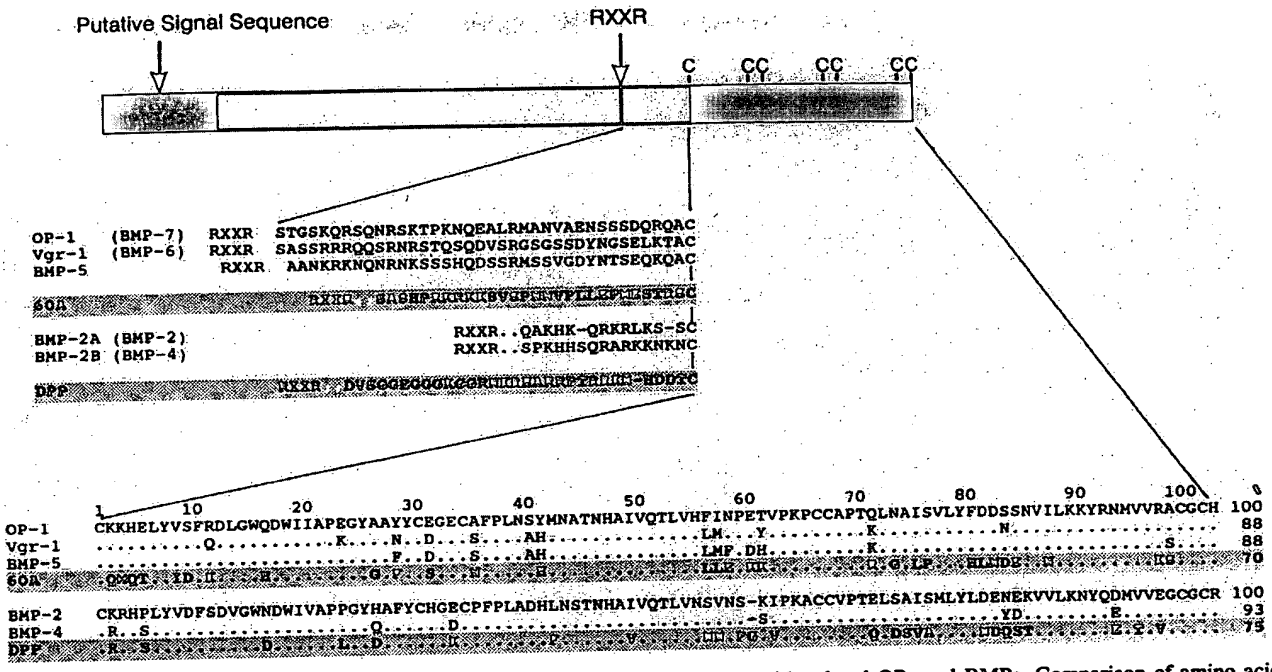

FIG. 1. Amino acid alignment of the mature *Drosophila* dpp and 60A proteins with related OPs and BMPs. Comparison of amino acid sequences within the conserved seven-cysteine domain (TGF-β domain) indicates that dpp is more closely related to BMP-2/4 (75% identity) and 60A is more closely related to BMP-5/6 and OP-1 (BMP-7) (70% identity). The degree of sequence similarity between dpp and 60A is considerably less (53%). The N-terminal extensions that precede the TGF-β domain, however, show considerably more evolutionary divergence among these proteins.

with the same buffer containing 100 mM NaCl, followed by 500 mM NaCl. The 500 mM NaCl fraction containing the active protein was sequentially dialyzed against water and 30% (vol/vol) acetonitrile/0.1% trifluoroacetic acid before being subjected to C_{18} reverse-phase HPLC as described (23). The fractions containing dpp or 60A, as determined by Western blot analysis (using affinity-purified dpp and 60A polyclonal antibodies, respectively) and by Coomassie blue staining, were pooled. The purity and concentrations of dpp and 60A proteins used for the evaluation of bone-forming activity were estimated by scanning at 580 nm the Coomassie blue-stained protein bands separated by SDS/PAGE (see Fig. 2). The concentration of dpp and 60A proteins was determined by comparison to a standard curve generated using known amounts of bovine serum albumin. A comparison of the absorbance by the bovine serum albumin band and a band containing known amount of standard OP-1 (previously quantitated by amino acid analysis) suggested that this approach is feasible for this family of proteins.

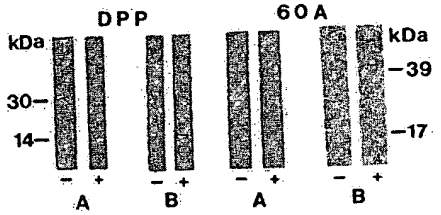

FIG. 2. Purification and characterization of recombinant *Drosophila* dpp and 60A proteins. Lanes: A, Coomassie blue staining of intact (−) and reduced (+) forms of dpp and 60A; B, Western blots using BMP-2-specific antiserum (for dpp) and OP-1-specific antisera (for 60A). Note BMP-2 antiserum is able to detect both intact and reduced forms of dpp, whereas OP-1 antiserum detects only the reduced form of 60A.

In Vivo Assay of Bone-Forming Activity. Highly purified *Drosophila* dpp or 60A protein (see Fig. 2) or recombinant human OP-1 (23) was reconstituted with demineralized guanidine hydrochloride-extracted rat collagen carrier by the 50% acetonitrile/0.1% trifluoroacetic acid lyophilization method (23) and implanted in a subcutaneous site in the thorax region of 28- to 35-day-old male Long–Evans rats. In brief, 25 mg of demineralized 4 M guanidine hydrochloride-extracted rat collagenous bone matrix (rat collagen carrier) was added to various concentrations of protein dissolved in 200 μl of 50% acetonitrile/0.1% trifluoroacetic acid that was then vortex-mixed and, subsequently, lyophilized. Rat collagen carrier alone was the negative control. The day of implantation was designated as day 0 of the assay. Implants were removed on days 7, 12, and 21 for histological evaluation. Bone-forming activity in the day 12 implants was monitored by the specific activity of alkaline phosphatase or calcium content of the implant (2). Values are the average of four to six implants from two or three rats. For histological examination, implants were fixed in Bouin's solution, embedded in JB4 plastic medium,

Table 1. Bone-inducing activity by recombinant *Drosophila* dpp and 60A proteins

Protein	Protein concentration, ng per implant	Alkaline phosphatase, units/mg of protein	Calcium content, μg/mg of tissue	Histology
dpp	—	0.06	ND	−
	480	1.43	ND	+++
	1440	1.48	ND	+++
60A	—	ND	1.95	−
	400	ND	9.75	++
	800	ND	15.20	+++
	1600	ND	19.60	+++

−, Absence of bone formation; ++, moderate bone formation; +++, extensive bone formation; ND, not determined.

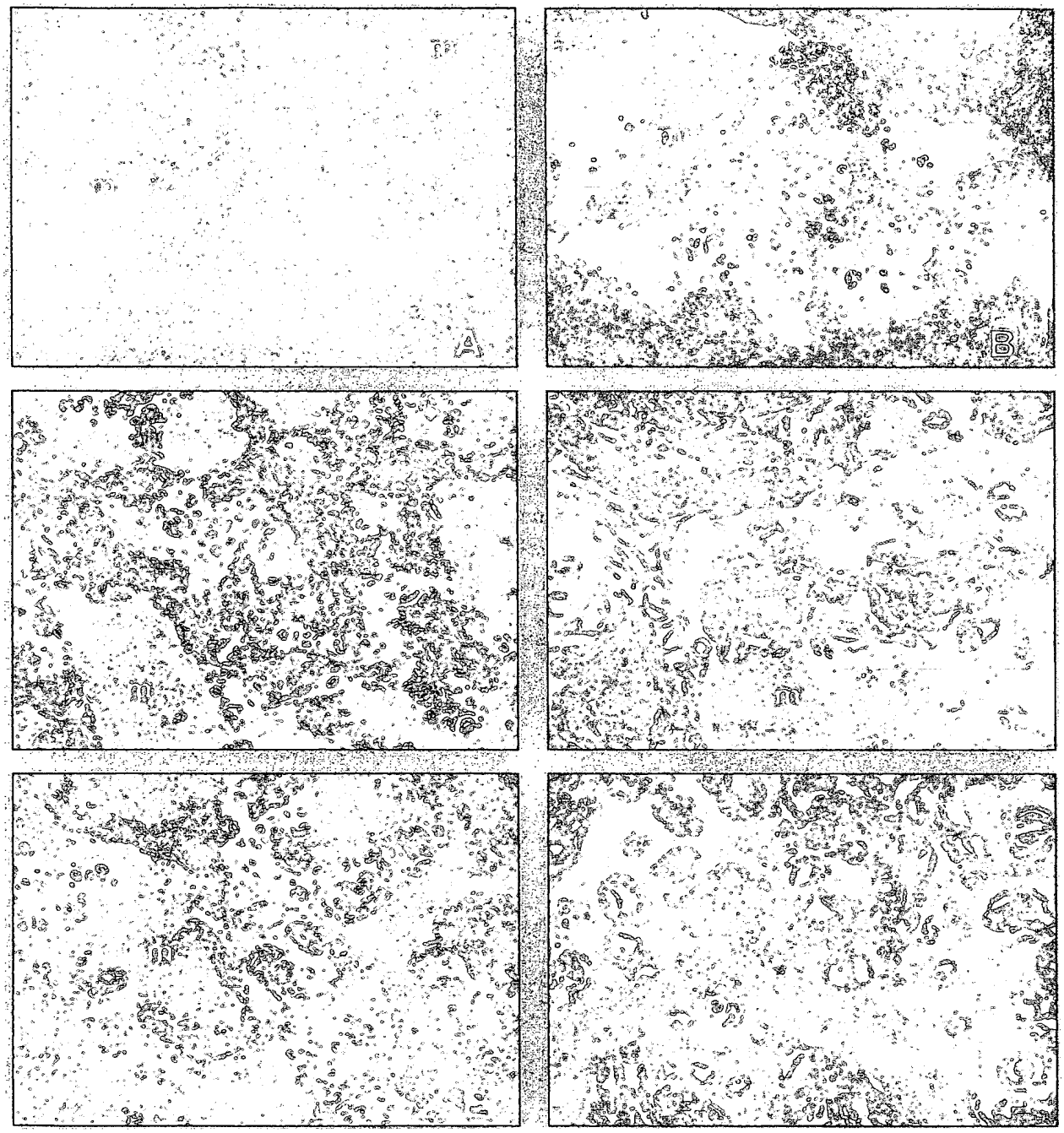

FIG. 3. Photomicrographs of histological sections of subcutaneous implants obtained from rats. (*A*) Negative control (day 12), guanidine hydrochloride-extracted rat demineralized bone matrix (m) (rat carrier). Note the absence of new bone formation. The implant consists of bone matrix and surrounding mesenchyme. (×160.) (*B*) Rat carrier (25 mg) reconstituted with 1 μg of recombinant *Drosophila* dpp (day 7). Evidence of chondrogenesis is seen. Newly formed cartilage cells, chondroblasts, and chondrocytes (Cy) are seen in close contact with the rat carrier matrix (m). A similar response was also seen in the *Drosophila* 60A-containing implants (data not shown). (×180.) (*C* and *D*) Rat carrier (25 mg) reconstituted with 2 μg of recombinant *Drosophila* dpp (day 12). Note evidence of endochondral bone formation (e.g., cartilage calcification, hypertrophy of chondrocytes, vascular invasion, and the onset of new bone formation). Arrows indicate the osteoblasts in close proximity with the vascular endothelium (v). Signs of remodeling are already apparent as shown by the presence of multinucleated osteoclasts. Also, there are early signs of bone marrow recruitment (Bm) in the newly formed ossicles. (*C*, ×220; *D*, ×290.) (*E* and *F*) Rat carrier (25 mg) reconstituted with 1.2 μg of *Drosophila* 60A protein (day 12). Note evidence of endochondral bone formation. The newly formed bone matrix deposited by osteoblasts is extensively mineralized and filled with numerous osteocytes (Oy). Signs of chondrolysis and bone remodeling are evident. (*E*, ×180; *F*, ×290.)

cut into 1-μm sections, and stained with toluidine blue (American HistoLab, Gaithersburg, MD).

Production of Antibodies. The cDNA clones that encode the mature C-terminal region of the human OP-1 gene (8) (aa 293–431, ≈14 kDa) and of the human BMP-2 gene (5) (aa 282–396, ≈13 kDa) were expressed as fusion proteins, respectively, in *Escherichia coli* (22). The OP-1 or BMP-2 fusion proteins, which were produced intracellularly as in-

clusion bodies, were solubilized and cleaved using mild acid to release the leader peptide. After purification, the human OP-1 or human BMP-2 polypeptides were used to raise polyclonal antibodies in rabbits. Antisera were tested for reactivity to intact and reduced bovine OP preparations (highly purified bovine OP preparations were found to be composed of dimers of OP-1 and BMP-2) (22) by Western blot analyses (data not shown). Antibodies to the *Drosophila* 60A protein were prepared against fusion proteins and used for Western blot analysis, as described (21). Antibodies specific to the C-terminal portion of dpp were generated against a glutathione *S*-transferase fusion protein containing the C-terminal 132 aa of dpp. The fusion protein was solubilized from inclusion bodies, purified by SDS/PAGE, and injected into rabbits by methods identical to those used for the generation of antisera against 60A (21).

Analytical Methods. Protein fractions were characterized by SDS/PAGE on 15% mini gels (0.5 mm thick) with a 3% stacking gel (22). Samples dissolved in Laemmli sample buffer were heated in boiling water for 3 min with or without dithiothreitol (100 mM) prior to electrophoresis. For Western blot analysis, samples subjected to SDS/PAGE were transferred to Immobilon membranes (Millipore) and incubated with specific rabbit antisera and, subsequently, with goat anti-rabbit immunoglobulin-linked peroxidase. Amino acid sequence analysis was performed using an Applied Biosystems protein/peptide sequencer, as described (22).

RESULTS AND DISCUSSION

Full-length *Drosophila* dpp and 60A cDNA clones expressed in insect cells yielded correctly processed mature disulfide-linked dimeric protein. The recombinant proteins were purified from either extracts of the proteins adhering to the tissue culture plate (dpp) or from the conditioned culture medium (60A). As is the case with other members of the TGF-β superfamily, the *dpp* and *60A* gene products are synthesized as precursors that are approximately three times larger than the processed mature disulfide-linked dimeric proteins. The purity of the mature dpp and 60A proteins was confirmed by N-terminal amino acid sequence analyses, Coomassie blue staining, and Western blot analyses after SDS/PAGE under nonreducing and reducing conditions (see Fig. 2). The Coomassie-stained reduced dpp protein (16 kDa) and 60A protein (18 kDa) excised from the Immobilon membrane were used for N-terminal amino acid sequence analysis. The N termini of the purified proteins (DVSGGEGGGKGG, for dpp, and XAXHPRKRKKSV, for 60A) corresponded to the sequences of the predicted proteolytic processing sites of the mature dimeric proteins (Fig. 1). Western blot analyses of purified dpp and 60A proteins showed they reacted specifically with their respective antisera (data not shown). In addition, examination of cross-reactivity by Western blots demonstrated that both intact and reduced dpp reacted with human BMP-2 antisera with a similar intensity. 60A protein, however, reacted with human OP-1 antisera weakly under reducing conditions and did not react under nonreducing conditions (Fig. 2).

Evaluation of the bone-forming activity of recombinant *Drosophila* dpp protein and 60A protein in subcutaneous rat implants harvested on days 7, 12, and 21 indicated that both induce bone formation (via cartilage as intermediate tissue), which is subsequently remodeled and filled with functional bone marrow elements (Table 1 and Fig. 3). The sequence of cellular events is comparable to that exhibited by demineralized bone matrix implants. Both dpp and 60A proteins induced cartilage formation, determined by histology of the day 7 implants (Fig. 3*B*). Bone formation induced by dpp protein or 60A protein was associated with cartilage hypertrophy, cartilage calcification, and vascular invasion. Day 12 implants containing 1–2 μg of either dpp or 60A showed bone remodeling and early signs of bone marrow recruitment (Fig. 3 *C* and *E*). Bone-forming activity by dpp protein and 60A protein was also demonstrated by determining the specific activity of alkaline phosphatase or calcium content of day 12 implants (Table 1). Evaluation of day 18–21 implants showed evidence of further remodeling and formation of ossicles filled with bone marrow elements (data not shown). In the absence of dpp or 60A protein, the collagen carrier implants recruited mesenchymal cells and did not show any sign of cartilage or bone formation (Fig. 3*A*). Bone-inducing activity by dpp protein and 60A protein is reproducible and is exhibited only by the column fractions that contained either protein. The degree of response was dependent on the dose of dpp or 60A protein contained in the implants. Since extensive dose curves were not performed at various time intervals, we are unable to directly compare the specific bone-inducing activities of the dpp protein or 60A protein relative to those of OP-1 or BMP-2. However, the present study shows that the concentrations of dpp or 60A protein required to induce bone formation are within the dose ranges that have been reported for recombinant human BMP-2 and recombinant human OP-1 proteins (23, 24).

These insect proteins produced by insect cells were biologically active in mammals, as shown by their ability to initiate the same cellular and developmental responses as the related mammalian protein. The specific responses to the insect proteins were probably dictated by the microenvironment at the implant site and the developmental potential of the responding cells. Whereas bone formation was stimulated by either dpp protein or 60A protein in the present study, it is possible that other tissues will have specific, but different, responses to various BMPs. It is worth noting that the major sites for synthesis of OP-1 and BMP-4 mRNA are the kidney and lung, respectively (8). Recently, OP-1 was shown to induce the neural cell adhesion molecule in a neuroblastoma–glioma hybrid cell line (28). Neural cell adhesion molecule has been shown to play a fundamental role in the development and regeneration of the nervous system. It will be of interest to determine whether specific OPs/BMPs are sufficient to induce tissue regeneration in other tissues. The understanding of how tissues respond to the BMPs, the signal transduction processes caused by receptor activation, and the phenotypic change in different tissues will provide additional insight into the role of BMPs in tissue formation, regeneration, and repair. The functional homology of the ligands investigated in the present study suggests that BMP-mediated instruction of pluripotent cells to pursue specific developmental fates has been well conserved during evolution.

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Developmental Biology

Human BMP sequences can confer normal dorsal–ventral patterning in the *Drosophila* embryo

(decapentaplegic/human BMP4/embryonic rescue/chimeric genes)

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ABSTRACT The type β transforming growth factor family is composed of a series of processed, secreted growth factors, several of which have been implicated in important regulatory roles in cell determination, inductive interactions, and tissue differentiation. Among these factors, the sequence of the DPP protein from *Drosophila* is most similar to two of the vertebrate bone morphogenetic proteins, BMP2 and BMP4. Here we report that the human BMP4 ligand sequences can function in lieu of DPP in *Drosophila* embryos. We introduced the ligand region from human BMP4 into a genomic fragment of the *dpp* gene in place of the *Drosophila* ligand sequences and recovered transgenic flies by *P*-element transformation. We find that this chimeric *dpp–BMP4* transgene can completely rescue the embryonic dorsal–ventral patterning defect of null *dpp* mutant genotypes. We infer that the chimeric DPP–BMP4 protein can be processed properly and, by analogy with the action of other family members, can activate the endogenous DPP receptor to carry out the events necessary for dorsal–ventral patterning. Our evidence suggests that the DPP–BMP4 signal transduction pathway has been functionally conserved for at least 600 million years.

The type β transforming growth factor (TGF-β) family is composed of several members of a secreted family of polypeptides that have profound effects on cell growth and differentiation (1–6). Their effect on cells occurs through binding to specific serine/threonine kinase receptors that transduce signals that alter the expression of downstream genes. The developmental effects of this growth factor family cover a wide spectrum but are usually associated with negative growth control.

In *Drosophila*, there are two known TGF-β-like members, the *dpp* gene and the *60A* gene. The *dpp* ligand acts in a signal transduction pathway to establish the identity of dorsal ectoderm in the early embryo. Later in development, the DPP protein is involved in other morphological events, such as visceral mesoderm formation and disk development. Mutations in the *60A* gene have not been identified, but its expression pattern suggests a role in embryonic mesoderm and ectoderm determination.

All nascent polypeptides of the TGF-β family members that have been studied are proteolytically processed to produce a propeptide and a mature polypeptide. The C-terminal mature region (in dimeric form) is the bioactive part of the molecule that binds the appropriate cellular receptors. It is the mature region, typically 110–130 amino acids long, that contains the seven invariant cysteine residues characteristic of all family members. The similarity of *dpp* to other TGF-β-like molecules follows a continuum, with the human BMP2/BMP4 proteins being most similar. DPP and BMP2/BMP4 are 75% identical over the C-terminal 100 amino acids of the mature region (Fig. 1*A*) and ≈30% identical in the propeptide region (7). Besides these high levels of sequence conservation, the localization of three *dpp* point mutations that disrupt all phenotypes controlled by *dpp* indicates that this C-terminal 100-amino acid domain is required for all *dpp* functions (K. Wharton, R. Ray, and W.M.G., unpublished results).

We have begun to examine whether the extensive structural similarity reflects functional conservation of DPP to BMP2/BMP4. We are defining functional conservation to mean a mechanistic conservation rather than a common set of cells or tissues affected by these protein factors. Presumably, the developmental potential of the cell will determine the consequences of signaling by these growth factor ligands, as is true for the effects *dpp* has on the developing *Drosophila*. For this purpose, we have used the earliest requirement for the *dpp* gene, determination of embryonic dorsal ectoderm (8–10), as a bioassay. We have constructed chimeric molecules and asked whether they are able to rescue the dorsal ectoderm of animals lacking *dpp*.

MATERIALS AND METHODS

Generation of Molecular Constructs. To facilitate construction of the chimeric genes, restriction sites were introduced into the appropriate clones. *Nar* I and *Sca* I restriction sites were introduced into *dpp* or *BMP4* by site-directed mutagenesis using mutant oligonucleotides (11). A *Nar* I site was introduced after the first conserved cysteine in the C terminus of the mature ligand region. The *Sca* I site was introduced after the termination codon of the protein. The introduction of new restriction sites did not change any of the amino acids encoded by either gene. The entire mutagenized insert was sequenced to verify that no unwanted mutations were introduced during the *in vitro* manipulations. The remainder of the coding region outside the *Nar* I and *Sca* I sites is derived from the *Hin* region of the *Drosophila dpp* gene. An 8-kb fragment containing the *dpp–BMP4* chimeric gene was cloned into the *P*-element transformation vector CaSpeR (12).

***Drosophila* Strains and Manipulations.** *dppH61* is a small deficiency that removes most of the 3′ coding exon of *dpp* (13) and *Df(2L)DTD48* is a deficiency for all of *dpp* (14). *SM6a* is a balancer for the second chromosome (15). Strains containing multiple copies of the chimeric transposon were generated by transposing the construct onto the desired chromosome with a strain containing an active transposase (16) or by recombining multiple copies onto a single chromosome. Germ-line transformants were obtained by standard techniques (17). Cuticle preparations were done on embryos as described (18).

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Abbreviation: TGF-β, type β transforming growth factor.

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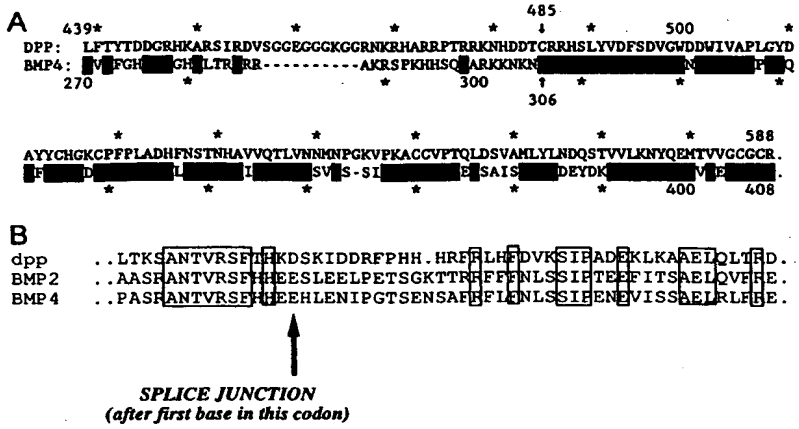
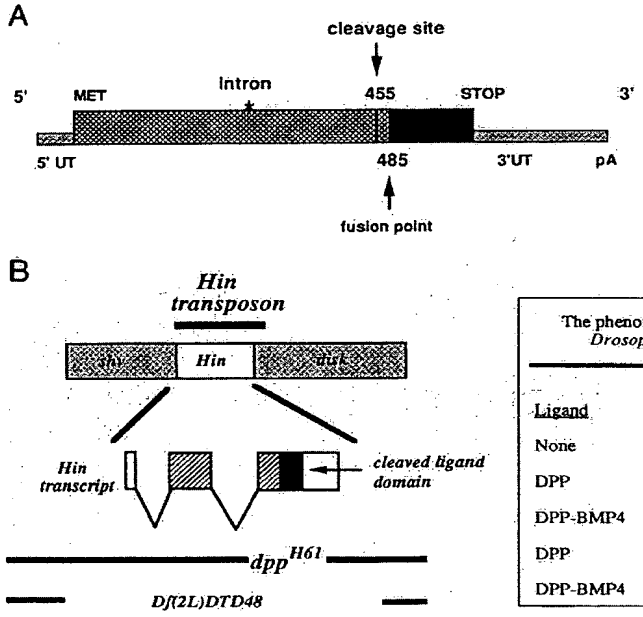


FIG. 1. Relationship of *dpp* and *BMP4*. (*A*) C-terminal alignment of DPP and BMP4 polypeptides. Solid boxes in place of amino acid sequences indicate perfect matches between the two factors. Asterisks denote increments of 10 amino acids in the sequence. (*B*) Alignment of amino acid sequences around the common intervening sequence. Shown are sequences on both sides of the introns of the *dpp* and *BMP4* genes, which allow for unambiguous placement of the intron.

RESULTS AND DISCUSSION

Conservation of Gene Structure. In addition to the sequence similarities of the DPP and BMP2/BMP4 polypeptides, there are considerable parallels between their transcripts. While the *dpp* gene (>55 kb) produces several transcripts, each has a similar structure (8). Each transcript consists of a unique 5′ untranslated exon together with common middle and 3′ exons. Because the open reading frame is entirely contained within the middle and 3′ exons, all *dpp* transcripts encode the identical polypeptide (R.W.P. and W.M.G., unpublished results). BMP2 and BMP4 have 5′ noncoding exons as well. Furthermore, like *dpp*, the coding regions of BMP2 and BMP4 are contained in two exons (J.M.W., unpublished results). The position of the intron between the two coding exons is in exactly the same position for all three genes. This is apparent by examining the alignment of protein sequences around this intron of the three genes (Fig. 1*B*). Sequences just upstream (ANTVRSF) and downstream (AELQ/R) of the splice sites for *dpp* and BMP2/BMP4 permit an exact alignment of the proteins in this region. Strikingly, the splice sites are at identical positions, after the first base in the fourth



The phenotypes of *Df(2L)DTD48/ dpp^{H61}* embryos carrying *Drosophila dpp* or human BMP4 chimera transgenes.

Ligand	Transgene copy number	Embryonic phenotype
None	0	strong ventralization
DPP	1	weak ventralization
DPP-BMP4	2	weak ventralization
DPP	2	normal
DPP-BMP4	3	normal

FIG. 2. *dpp–BMP4* chimera rescue experiments. (*A*) Structure of the mRNA derived from the *dpp–BMP4* chimeric transgene. Solid box represents human BMP4 ligand sequences that were introduced into the chimeric construct as a replacement for comparable sequences of the DPP ligand. Fusion site of the DPP and human BMP4 polypeptides is at the first conserved cysteine in the C terminus (amino acid 485 in the DPP sequence and 306 in BMP4) (see arrows in Fig. 1*A*), 30 amino acids from the putative proteolytic cleavage site (20, 21). Other than this inclusion of DPP sequences, the ligand is totally derived from BMP4. Asterisk indicates position of the common intron between the middle and 3′ exons. *dpp* genomic sequences extend for 1 kb on the 5′ and 1.5 kb on the 3′ side of the *Hin* transcription unit. UT, untranslated region. (*B*) Summary of chimera rescue experiments. (*Left*) Position in the *dpp* gene where the sequences for the rescue are derived. Transposons that contain the entire *Hin* region of *dpp* are capable of rescuing animals to hatching (19). (*Right*) Mutant phenotypes obtained by different doses of wild-type *dpp* and chimeric transposons.

codon C-terminal to the ANTVRSF motif. The structural parallels between their processed transcripts further strengthen the argument that *dpp* and BMP2/BMP4 have a common evolutionary origin.

The Chimeric Gene Can Rescue *Drosophila* Embryos. To test whether the human BMP ligand sequences could substitute for *dpp* in early development, we chose to replace the *Drosophila dpp* sequences with the human counterpart. In *Drosophila*, the formation of the dorsal ectoderm offers a sensitive bioassay for function. The dorsal–ventral patterning function of *dpp* in embryonic development is contained within an 8-kb fragment spanning the centrally located *Hin* region of the *dpp* gene (19). This region includes the most abundant *dpp* embryonic transcription unit (6, 8) together with sufficient cis-regulatory sequences to confer expression of *dpp* in the dorsal ectoderm during blastoderm and germband extension stages (R.W.P. and W.M.G., unpublished data). If transgenic constructs produce sufficient amounts of correctly regulated *dpp* activity, animals completely lacking endogenous *dpp* gene activity will hatch to larvae and display a normal larval cuticular pattern. For the 8-kb *Drosophila Hin* region construct, two copies of the transgene typically fully rescue dorsal–ventral patterning in a *dpp* null genetic background. We chose to attempt the rescue of a *dpp* null animal with our chimeric transgene rather than to attempt to create gain-of-function phenotypes by ectopic expression, since rescue directly and stringently tests whether the transgene can fulfill the normal requirement for *dpp* activity in its signal transduction pathway.

We have introduced a chimeric *Hin* region rescue transgene, in which the bulk of the ligand region (amino acids 485–588) was derived from the human *BMP4* gene (Fig. 2*A*), using standard *P*-element-mediated germ-line transformation (17). In these experiments, we did not want to determine whether the C-terminal proteolytic processing was conserved between *Drosophila* and humans. For this reason, we selected amino acids 485–588 encoded by the human gene for the *dpp–BMP4* swap to ensure that we were not replacing the C-terminal sequences necessary for protease recognition and cleavage of the mature ligand from the propeptide region. If the chimeric protein was not properly processed, then we would not be able to test whether it was able to function in place of the endogenous *dpp* gene. The phenotypic effects of this chimeric transgene in dpp mutant backgrounds were compared to those of a *Hin* transgene composed solely of *Drosophila dpp* sequences.

We have observed functional dpp^+ activity of the chimeric constructs in several genotypes with reduced or no endogenous *dpp* activity. For example, a single copy of the chimeric transgene is sufficient to rescue fully the otherwise haplolethal $dpp^+/Df(2L)dpp$ genotype (data not shown). The most striking demonstration of the activity of the chimeric transposon comes from genotypes totally lacking endogenous *dpp* activity (Figs. 2*B* and 3; Table 1). This genotype is heterozygous for two *dpp* deletions: a large one in which the entire chromosomal region surrounding *dpp* has been removed [*Df(2L)DTD48*] and a small one in which most or all of the 3′ exon, including the ligand coding region, is missing (dpp^{H61}). In such a *dpp* null background, partial and full rescue of

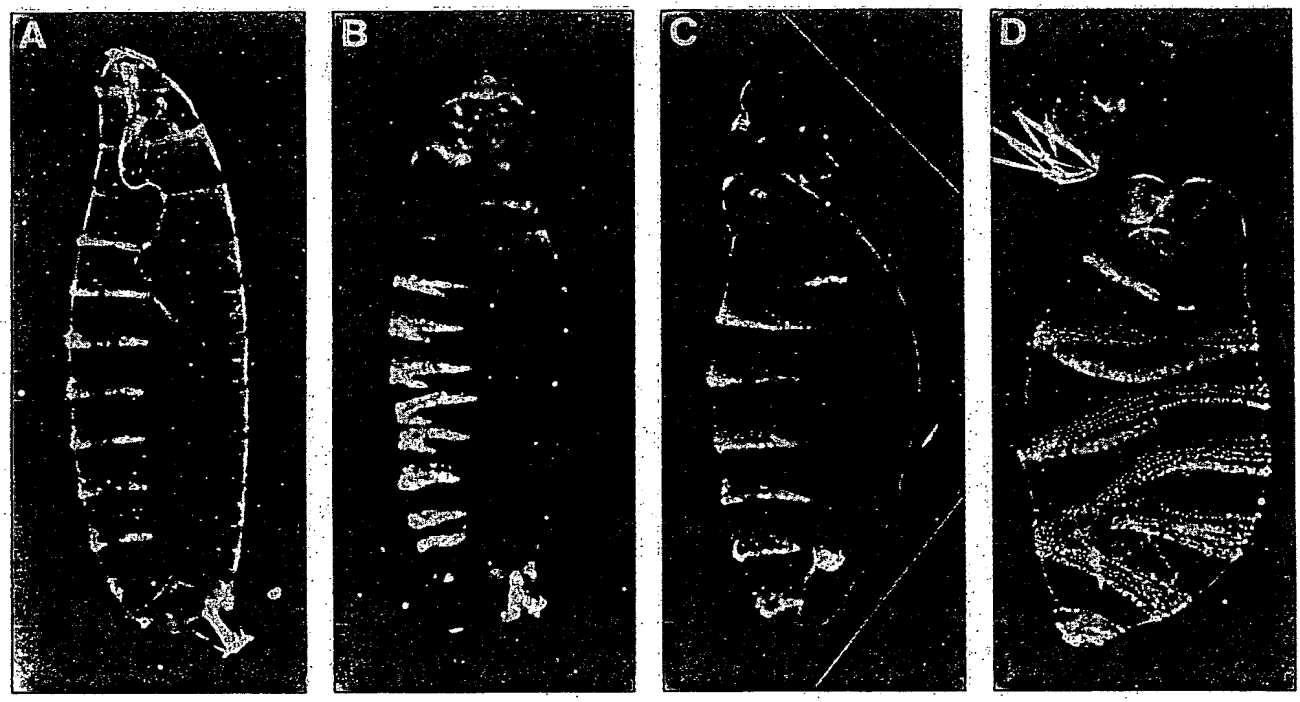


FIG. 3. Dark-field micrographs of cuticle preparations of wild-type and transgenic embryos. Dorsal is on top and anterior is on the left. All animals were derived from crosses that resulted in offspring containing the dpp^{H61} and *Df(2L)DTD48* null *dpp* alleles and different numbers of transposons as indicated. Rescuing activity can be observed by comparing the mutant phenotype of a *dpp* null allele (*D*) with different doses of the transposon (*A–C*). (*A*) Phenotype of a wild-type larva. The cuticle of this larva is indistinguishable from one mutant for dpp^{H61}/*Df(2L)DTD48* and harboring three or four copies of the chimeric transgene. (*B*) Mutant phenotype of a dpp^{H61}/*Df(2L)DTD48* embryo containing two copies of the transgene. Note that the primary defects of these embryos reside in the head and terminal region, reminiscent of mutant phenotypes seen in weak *dpp* alleles. (*C*) Mutant phenotype of an embryo containing one copy of the transposon. Note the more severe reductions in cephalic structures and the posterior filzkörper relative to *B*. (*D*) Mutant phenotype of a *dpp* null embryo. This dpp^{H61}/*Df(2L)DTD48* embryo lacks all endogenous *dpp* function and contains no transgene. This results in the complete absence of dorsal epidermis; there is a concomitant expansion of the ventral epidermal domain around the entire circumference of the embryo.

Table 1. Crosses to determine effects of the chimeric transgene on rescue of *dpp* null genotypes

Cross	Cross to generate test embryos		Resulting progeny*	
	♀ parent	♂ parent	Total fertilized eggs	% dead eggs
1	dpp^{H61}/*CyO-P23*†	*Df(2L)DTD48/CyO-P23*	378	46‡
2	dpp^{H61} *TnA*§/*SM6a*	*Df(2L)DTD48 TnB*/+	1480	24¶
3	*Df(2L)DTD48 TnC/SM6a*	dpp^{H61} *TnA*/+	621	29
4	dpp^{H61} *TnA TnD/SM6a*	*Df(2L)DTD48 TnB*/+	304	1
5	dpp^{H61} *TnA TnD/SM6a*	*Df(2L)DTD48 TnC TnD*/+	504	4
6	dpp^{H61} *TnA TnE/SM6a*	*Df(2L)DTD48 TnC TnD*/+	1379	6

*Embryos from brief egg lay collections were placed on medium-containing grids. Unfertilized eggs (white eggs) and dead embryos (discolored eggs) were counted 24–36 hr later.

†dpp^{H61} and *Df(2L)DTD48* are haplolethal. To create viable balanced strains containing these mutations, a transgene containing a second copy of the *dpp Hin* region was transposed onto the standard *CyO* balancer. This derived balancer, *CyO-P23*, contributes sufficient dpp^{+} activity to rescue balanced *dpp* null alleles.

‡Approximately one-half of the dead embryos from cross 1 are dpp^{H61}/*Df(2L)DTD48*. The others are presumably *CyO-P23* homozygotes.

§In crosses 2–6, each *dpp* null-bearing second chromosome also contains one or more copies of the chimeric transgene. Five different independent insertions of the transgene were used (designated *TnA–TnE*).

¶In crosses 2–6, the male parent was heterozygous for a wild-type (i.e., dpp^{+}) chromosome. Thus, the only progeny class that could potentially give rise to a large proportion of inviable eggs would be the dpp^{H61} *Tn*/*Df(2L)DTD48 Tn* offspring. Hence, if this genotype were totally inviable in one of the crosses, 25% dead embryos are expected; this result obtains in crosses 2 and 3. If the genotype is totally viable, essentially no dead embryos are expected; this result obtains in crosses 4–6.

embryonic dorsal–ventral pattern is achieved by one and two copies, respectively, of the *Drosophila* transgene (19). In contrast, the chimeric *dpp–BMP4* construct is less efficient at rescuing this genotype. One or two copies of the chimeric *dpp–BMP4* transgene only achieves partial rescue. To determine whether this partial rescue were simply a quantitative effect or an intrinsic limitation of the chimeric transgene, genotypes containing three or four copies were then tested. Full rescue of the embryonic dorsal–ventral pattern is achieved with both three and four copies of the chimeric transgene as indicated by the proportion of dpp^{-} individuals that hatch (Table 1; Fig. 3). Thus, we conclude that, in the complete absence of endogenous dpp activity, a sufficient level of active chimeric DPP–BMP4 ligand can be generated to lead to a wild-type level of activation of the DPP receptor.

Why is the response of a given dosage of the chimeric transposon less robust than the comparable dosage of the *Drosophila dpp* transgene? Our preferred model is that the human ligand may have a lower affinity for the DPP receptor. The residues that bind to the receptor have not been positively identified so we cannot examine this possibility. One-fourth of the amino acids in the chimera are different from those of DPP and we know that flies are sensitive to the dose and activity of the *dpp* gene. There are other reasonable possibilities to account for the reduced activity of the chimeric gene. For example, it may be that we have altered the rate of proteolytic processing (in spite of our choice for the fusion site between the two genes) since these sites are not well defined. It may be that the DPP propeptide region or some other factor [e.g., tolloid (22)] does not interact with the BMP4 ligand region as efficiently, thus reducing the amount of active protein (23). Our present assay systems are incapable of distinguishing among these possibilities.

We have made a similar chimera between *dpp* and *BMP2*. While not yet tested for its ability to rescue homozygous null *dpp* genotypes, we have found that the one copy of the *dpp–BMP2* chimera fully rescues *dpp* monosomics (that are otherwise inviable), just as the *dpp–BMP4* chimera does (data not shown).

CONCLUDING REMARKS

The results of these studies are significant for several reasons. A secreted protein from humans has been shown to function in invertebrates. Most protein swapping experiments have shown conserved function of transcription factors in heterologous systems (24, 25). Furthermore, our experiments required that normal levels of the protein rescue a mutant phenotype. Most other gene replacement experiments involve overexpression of the heterologous genes to achieve a mutant phenotype, a less stringent criterion of conserved function. The similar structures of the *dpp* and *BMP2/BMP4* transcription units, in conjunction with functional conservation, add strength to the argument that these genes are true evolutionary homologs in the arthropod and vertebrate lineages, respectively.

Given strong arguments for evolutionary and functional conservation of these genes, it may be that they have retained common developmental roles (4, 5, 13, 26, 27). At present, this is difficult to determine. Since it is hard to equate the embryological events between *Drosophila* and vertebrates with our current knowledge, it is difficult to assign a similar developmental function to *dpp*, *BMP2*, and *BMP4*. Furthermore, both of these related human genes are involved in several uncharacterized developmental events, adding further difficulty to correlating developmental functions. However, it is clear from our study that these growth factors do not function to produce one kind of developmental event but rather send cellular signals that are interpreted in the context of the developmental state of the cell. This aspect of this growth factor family has been conserved for at least 600 million years.

The secreted proteins in the TGF-β family are involved in many protein–protein interactions prior to binding their respective cellular receptors (28–31). These protein interactions may be important for processing and/or altering the activity of the growth factor proteins, such as the role postulated for the tolloid/BMP1 proteins (7, 22). Receptors for DPP or any BMP have not been identified to date. However, given the nature of the structural conservations between all TGF-β family members, it is most plausible to expect that DPP and the BMPs act by binding to a transmembrane receptor, thereby initiating a signal transduction cascade. Since the chimeric DPP–BMP4 protein functions in *Drosophila*, then it must be folded properly, processed properly, bound in protein complexes, and presented to the receptor in a manner very similar to the endogenous *Drosophila* protein. Because this experiment was successful, it seems likely that many of the components of the signal transduction pathway have been conserved between *Drosophila* and vertebrates. These studies indicate that insights

we gain by studying these secreted factors in one system can likely be applied to other systems.

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APPENDIX II

DESIGN AND OPTIMIZATION OF DOSAGE REGIMENS; PHARMACOKINETIC DATA

Leslie Z. Benet, Svein Øie, and Janice B. Schwartz

The objective of this appendix is to present pharmacokinetic data in a format that allows the clinician to make rational choices of doses of drugs. Table A-II-1 contains quantitative information about the absorption, distribution, and elimination of drugs and the effects of disease states on these processes, as well as information about the correlation of efficacy and toxicity with measured concentrations of drugs in plasma. The general principles that are used to select the appropriate maintenance dose and dosing interval (and, where appropriate, the loading dose) for the average patient are described in Chapter 1. Discussion of individualization of these variables for a particular patient is presented here.

To use the data that are presented, one must understand clearance concepts and their application for the computation of drug-dosage regimens. One must also know average values of clearance, as well as some measures of the extent and kinetics of drug absorption and distribution. The text below defines the eight basic parameters that are listed in the tabular material for each drug, as well as some factors that influence these values both in normal subjects and in patients with particular diseases.

It would obviously be most useful if there was a consensus about the correct value for a given pharmacokinetic parameter, rather than being faced with 10 or 20 separate (and often disparate) estimates of that parameter. Unfortunately, a consensus has been reached for only a very limited number of drugs. In Table A-II-1, single values for each parameter and for its variability (standard deviation) in the population have been selected from the literature, based on the scientific judgment of the authors. Values in the tables are those determined in healthy normal adults, unless otherwise indicated in footnotes. The direction of change for these values in particular disease states is noted next to the average value. A single reference is included for each drug. In most cases, the reference is to a recent paper or review of the clinical pharmacokinetics, which can then serve as a source of other important papers to which the reader also may refer.

TABULATED PHARMACOKINETIC PARAMETERS

Each of the eight parameters presented in Table A-II been discussed in detail in Chapter 1. The followin cussion focuses on the format in which the values ai sented, as well as on factors (physiological or path cal) that influence the parameters.

Availability. The extent of availability of the dru oral administration is expressed as a percentage of th This value represents the percentage of an oral do is available to produce pharmacological actions—th tion of the oral dose that reaches the arterial bloo active form. *Fractional availability (F)* is a simila meter used elsewhere in this appendix; this value from 0 to 1. Measures of the *rate* of availability provided in Table A-II-1. Since pharmacokinetic cc are most useful in the design of multiple dosage reg the *extent* rather than the rate of availability is mor cal to obtain an appropriate concentration of drug body (*see* Chapter 1).

It is important to keep in mind that poor patie pliance may be mistaken for decreased bioavailab true decrease in bioavailability may result from a formulated dosage form that fails to disintegrate solve in the gastrointestinal fluids, interactions b drugs in the gastrointestinal tract, metabolism of tl in the gastrointestinal tract, and/or first-pass hepa tabolism or biliary excretion (*see* Chapters 1 and patic disease in particular may cause increased a ity because hepatic metabolic capacity decreases because of the development of vascular shunts aro liver.

Urinary Excretion of Unchanged Drug. The sec rameter in Table A-II-1 is the amount of drug ev excreted unchanged in the urine, expressed as a age of the administered dose. Values represent centage expected in a healthy young adult (creatini

ance greater than 100 ml/min). When possible, the value listed is that determined after bolus intravenous administration of the drug, at which time availability is assumed to be 100%. If the drug is given orally, this parameter may also reflect loss of drug because of low availability; such values are indicated in a footnote.

Renal disease is the primary factor that causes changes in this parameter. This is especially true when alternate pathways of elimination are available; thus, as renal function decreases, a greater fraction of the dose is available for elimination by other routes. Since renal function generally decreases as a function of age, the percentage of drug excreted unchanged also usually decreases with age when alternate pathways of elimination are available. For a number of acidic and basic drugs with values of pK_a in the range of the usual pH of urine, changes in the latter will affect the rate or extent of urinary excretion (*see* Chapter 1).

Binding to Plasma Proteins. The tabulated value is the percentage of drug in the plasma that is bound to plasma proteins at concentrations of the drug that are achieved clinically. In almost all cases the values are from measurements performed *in vitro* (rather than from measurements of binding to the proteins in plasma that were obtained from patients to whom the drug had been administered). When a single mean value is presented, there is no apparent change in this percentage over the range of concentrations normally found in patients taking the drug. In cases in which saturation of binding is approached at usual plasma concentrations, values are provided at concentrations that correspond to the lower and upper limits of the usual range.

Plasma protein binding is primarily affected by disease states (such as hepatic disease) that alter the concentration of albumin or other proteins in plasma that bind drugs. Some metabolic states and conditions, such as uremia, also change the affinity of binding for some drugs. Such changes in protein binding as a function of disease can dramatically affect the volume of distribution of a drug.

Clearance. Total systemic clearance of drug from plasma (*see* equation 3, Chapter 1) is given in Table A-II-1; values are usually reported in units of ml $\cdot$ min^{-1} $\cdot$ kg^{-1}. In some cases, separate values for renal and nonrenal clearance are also provided. For some drugs, particularly those that are predominantly excreted unchanged in the urine, equations are given that relate total or renal clearance to creatinine clearance (also expressed as ml $\cdot$ min^{-1} $\cdot$ kg^{-1}). For drugs that exhibit saturation kinetics, K_m and V_m are given and represent, respectively, the plasma concentration at which half of the maximal rate of elimination is reached (in units of mass/volume) and the maximal rate of elimination (in units of mass $\cdot$ $time^{-1}$ $\cdot$ kg^{-1} of body weight). The concentration of the drug in plasma (C_p) must, of course, be in the same units as K_m.

As discussed in Chapter 1, intrinsic clearance from blood is the maximal possible clearance by the organ responsible for elimination when blood flow (delivery) of drug is not limiting. Intrinsic clearance is tabulated for a few drugs. Note that intrinsic clearance is defined in terms of the concentration of drug in *blood*. If one wishes to relate changes in elimination of drug to pathological changes either in the organ itself or to blood flow to the organ, it is necessary to express clearance with respect to concentrations of drug in blood rather than those in plasma. This requires measurement of concentrations in whole blood or knowledge of the distribution of drug between plasma and red blood cells; such information is currently limited, but when available is provided in a footnote. Clearances from plasma are presented in Table A-II-1, since these are most useful to relate dosage of drug to concentrations of drugs in plasma that have been determined previously to be effective or toxic.

Clearance can be determined only when the fractional availability F of the drug is known. Therefore, to be accurate, clearances must be determined after intravenous dosage. When such data are not available, the ratio of CL/F is given; values of this kind are indicated in a footnote. When a drug, or its active isomer for racemic compounds, is primarily a substrate for a particular cytochrome P450 isoform (as discussed in Chapter 1), this information is provided in a footnote.

Clearance varies as a function of body size and, therefore, is presented in the table in units of ml $\cdot$ min^{-1} $\cdot$ kg^{-1} of body weight. Although normalization to measures of size other than weight may sometimes be appropriate, weight is so convenient that this offsets any small loss in accuracy, especially in adults.

Volume of Distribution. The total body volume of distribution at steady state (V_{ss}) is given in Table A-II-1 and is expressed in units of liters/kg.

When estimates of V_{ss} are not available, values for V_{area} are provided (*see* Chapter 1). These values are obtained by dividing clearance by the terminal rate constant for elimination. V_{area} is a convenient and easily calculated parameter. However, unlike V_{ss}, this volume term varies when the rate constant for drug elimination changes, even though there has been no change in the distribution space. Because the clinician may wish to know whether a particular disease state influences either clearance or the distribution of the drug within the body independently, it is preferable to define volume in terms of V_{ss}, a parameter that is theoretically independent of changes in the rate of elimination.

As is the case for clearance, V_{ss} is usually defined in able in terms of concentration in plasma, rather than ood. If data were not obtained after intravenous adstration of the drug, a footnote will make clear that parameter determined, V_{ss}/F, contains a measure of ability.

Volume of distribution is primarily a function of body and values in the table are given in liters/kg. As men-d for clearance, normalization to measures of size r than weight may sometimes be appropriate.

-life. The time required for one-half of the amount ug in the body to be eliminated is provided. However, concentrations in plasma often follow a multiexpoial pattern of decline when this value is measured as ıction of time. The single number listed in each com-nt of the table corresponds to the rate of elimination describes the major fraction of total clearance of drug ı the body. In many cases this half-life may correspond ıe terminal log-linear rate of elimination. For a numof drugs, however, a more prolonged half-life may be rved at very low plasma concentrations when exely sensitive assay techniques are used. If this comnt accounts for only 10% of drug clearance, predic-; of steady-state concentrations of drug in plasma will ı error by only 10% if this longer half-life is ignored, ıatter how large its value. This is true because half-life function of both elimination and distribution, as dised in Chapter 1.

Half-life is also a measure of the approach to steady-: during a multiple dosing regimen and a measure of ımulation, as described in Chapter 1. That is, for a fixed iple dose interval, the patient will be at 50% of steady : after one drug half-life, 75% of steady state after two ; half-lives, 87.5% of steady state after three drug half-:, *etc.* Because pharmacokinetic analysis and therapeurug monitoring are based on measured plasma (or blood) :entrations, the clinician should know the half-life that predict accumulation in the patient. That is the appro-:e half-life to use in equations 1–18 and 1–19 (*see* Chap-) to predict steady-state concentrations. It is this multilosing or accumulation half-life that is given in the table.

Half-life is usually independent of body size, since it function of the ratio of two parameters, clearance and me of distribution, each of which is proportional to y size.

ctive and Toxic Concentrations. There is no general ement about the best way to describe the relationship veen the concentration of drug in plasma and its effect. ıy different kinds of data are presented in the literature, extraction of a single parameter or even a set of para-:rs is difficult. Furthermore, there may not be agreement as to which measures are most relevant. Footnotes are common in Table A-II-1 to indicate the meaning and relevance of these values; in many cases reference is made to a chapter in the text for discussion. This is particularly true for antimicrobial agents, since the effective concentration depends on the identity of the microorganism causing the infection.

The relationships between the concentration of drug in plasma and the effect of the drug are imperfect, as might be expected (*see* Chapters 2 and 4). Little information is available about the variation between individuals of receptor number or affinity or subsequent coupling to a response, or about the effects of disease states on these factors. For many drugs, the form of the relationship between effect and plasma concentration is unknown. Because the concentration of free drug determines the degree of effect, changes in protein binding due to disease may be expected to cause changes in the *total* concentration of drug associated with a desired or an unwanted effect. It is also important to realize that concentration–effect relationships are meaningful only at steady state or during the terminal log-linear phase of the concentration-versus-time curve, when the ratio of the drug concentration at sites of action to that in the plasma can be expected to remain constant over time. Thus, when attempting to correlate pharmacokinetics with pharmacodynamics, the time of distribution of drug to its site of action must be taken into account.

ALTERATIONS OF PARAMETERS IN THE INDIVIDUAL PATIENT

The values in Table A-II-1 represent mean values for populations of normal adults, and it may be necessary to modify them for calculation of dosage regimens for individual patients. The fraction of free drug (α) in a given patient must be known in order to compute a desired steady-state concentration. The fraction available (F) and clearance must also be estimated to compute a maintenance dose. To calculate the loading dose and to estimate half-life and dosing interval, knowledge of the volume of distribution is needed. The figures in the table and the adjustments apply only to adults unless specifically designated otherwise. Although the values may sometimes, with caution, be applied to children who weigh more than about 30 kg (after proper adjustment for size; *see* below), it is best to consult a textbook of pediatrics or other source for definitive advice.

For each drug, changes in the parameters occasioned by certain disease states are noted within the eight segments of the table. In most cases, a qualitative direction of

changes is noted, such as "↓ Hep," which indicates a significant decrease in the parameter in a patient with hepatitis. A reasonable quantitative translation is to multiply the value of the parameter by 0.5 for each applicable condition that is noted to decrease the parameter and to multiply it by 2 for each condition that is noted to increase the parameter. Such an adjustment can only be approximate; yet, since reliable data are limited, no better approach may be possible. The relevant literature should be consulted for more definitive quantitative information.

Protein Binding. Most acidic drugs that are extensively bound to plasma proteins are bound to albumin. Basic drugs, such as propranolol, are often bound to other plasma proteins (*e.g.*, α_1-acid glycoprotein). The degree of drug binding to proteins will differ in states that cause changes in the concentrations of the binding proteins. Unfortunately, among binding proteins only albumin is commonly measured. For drugs that are bound to albumin (*alb*), a patient's fraction of free drug (α_{pt}) can be approximated from the following relationship:

$$\alpha_{pt} = 1 \Big/ \left[\left(\frac{alb_{pt}}{alb_{nl}} \right) \left(\frac{1-\alpha_{nl}}{\alpha_{nl}} \right) + 1 \right] \qquad \text{(A–1)}$$

where alb_{nl} and α_{nl} refer to values of the concentration of albumin in plasma and the fraction of free drug in normal individuals, respectively. Use of this equation assumes that the molar concentration of drug is far less than that of albumin, that only one type of drug binding site is present on albumin, and that there are no cooperative binding interactions. Therefore, it cannot be exact. However, it is a reasonable approximation and, in the absence of actual measurement of the patient's fraction of free drug, can prove quite useful.

Clearance. Clearance must often be adjusted for alterations in renal function. The quantities required for this adjustment are the fraction of normal renal function remaining and the fraction of drug usually excreted unchanged in the urine. The latter quantity appears in the table; the former can be estimated as the ratio of the patient's creatinine clearance to a normal value (120 ml/min per 70 kg). If creatinine clearance has not been measured, it may be estimated from measurements of the concentration of creatinine in serum, using a number of different equations or nomograms. One such method is to estimate the fraction of normal renal function present (*rfx*) as the reciprocal of the patient's serum creatinine concentration, minus 0.01 for each year of age over 40. This is a crude estimate, but more accurate ones are seldom justified or necessary, since the whole process of adjustment of clearance is already approximate because of considerable unpredictable interindividual variation in clearance, which is independent of renal function. The following equation for adjustment of clearance uses the quantities just discussed:

$$rf_{pt} = 1 - fe_{nl}(1 - rfx_{pt}) \qquad \text{(A–2)}$$

where fe_{nl} is the fraction of drug excreted unchanged in normal individuals (*see* table). The renal factor (rf_{pt}) is the value that, when multiplied by normal total clearance (CL_{nl}) from the table, gives the total clearance of the drug adjusted for disturbances of renal function.

Example. Clearance of terbutaline in a patient with depressed renal function (creatinine clearance = 40 ml $\cdot$ min^{-1} $\cdot$ 70 kg^{-1}) may be estimated as follows:

$$\begin{aligned} rfx_{pt} &= 40 \text{ ml/min} \div 120 \text{ ml/min} = 0.33 \\ fe_{nl} &= 0.56 \text{ (}\textit{see}\text{ listing for terbutaline)} \\ rf_{pt} &= 1 - 0.56\ (1 - 0.33) = 0.62 \\ CL_{pt} &= CL_{nl} \cdot rf_{pt} \\ CL_{pt} &= (3.4 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1})\ (0.62) \\ &= 2.1 \text{ ml} \cdot \text{min}^{-1} \cdot \text{kg}^{-1} \end{aligned}$$

Clearance must also be adjusted for the size of the patient. For convenience, the figures in the table are normalized to weight. However, clearance of drug often varies in proportion to metabolic rate, which is best related to weight to the 0.75 power. To take this into account, the clearance figure in the table can be multiplied by the factor *wf*, instead of by weight:

$$wf = Wt_{nl}(Wt_{pt}/Wt_{nl})^{0.75} \qquad \text{(A–3)}$$

which for weight in kg (where Wt_{nl} = 70 kg) becomes:

$$wf = 2.9(Wt_{pt})^{0.75} \qquad \text{(A–4)}$$

Calculation of the weight factor seldom pays for itself by yielding significantly more accurate estimates. It should be done, however, for patients at extremes of size, especially the very obese.

If drug elimination is proportional to the free (rather than the total) concentration of drug in plasma, clearance is then further adjusted by multiplying it by the ratio of the normal free fraction to the free fraction of the patient, calculated as indicated previously.

All the adjustments to clearance should be applied simultaneously. That is, the figure in the table is multiplied by a weight factor (usually weight itself), *and* multiplied by the binding correction, if applicable, *and* multiplied by the renal correction factor, if applicable, *and*, finally, multiplied by the appropriate values of 0.5 and/or 2 for other factors that are present and qualitatively indicated to modify clearance. Obviously, if a quantitative correction is

ıade for renal function, a qualitative one (multiplication y 0.5) should not also be made for this same factor.

'olume of Distribution. Volume of distribution should e adjusted for the modifying factors indicated in Table ·-II-1, as well as for size. The figures in the table are nor-ıalized to weight. Unlike clearance, volume of distribu-on is probably most often proportional to weight itself. Vhether or not this is so, however, depends on the actual ites of distribution of drug, and no absolute rule applies.

Whether to adjust volume of distribution for changes n binding to plasma proteins cannot be decided in gen-ral, since the decision critically depends on whether the actors that alter binding to plasma proteins also alter bind-ng to tissues. In such cases the qualitative changes in vol-ıme of distribution are indicated in the table. Again, each ıdjustment to volume of distribution should be made in-lependently of any other, and the final estimate should re-lect all adjustments simultaneously.

Half-life. Finally, half-life may be estimated from the ad-usted estimates of clearance and volume of distribution:

$$t_{1/2} = 0.693\ V_{pt}/CL_{pt} \qquad \text{(A–5)}$$

Since, historically, half-life has been the parameter most often measured, qualitative changes for this parame-ter are almost always given in the table.

INDIVIDUALIZATION OF DOSAGE

By using the parameters for the individual patient, calcu-lated as described above, initial dosing regimens may bc chosen. The maintenance dose may be calculated with equa-tion 1–16 in Chapter 1, and the estimated values for CL anc F for the individual patient. The target concentration may need to be adjusted for changes in protein binding in thc patient, as described above. The loading dose may be cal-culated by use of equation 1–20 in Chapter 1 and the esti-mated parameters for V_{ss} and F. A particular dosing inter-val may be chosen; the maximal and minimal steady-statc concentrations can be calculated by using equations 1–18 anc 1–19 in Chapter 1, and these can be compared with the effi-cacious and toxic concentrations listed for the drug. As witl the target concentration, these values may need to be ad-justed for changes in the extent of protein binding. Use o equations 1–18 and 1–19 also requires estimates of value for F, V_{ss}, and k ($k = 0.693/t_{1/2}$) for the individual patient

Note that these adjustments of the pharmacokineti parameters for an individual patient are suggested for th rational choice of initial dosing regimen. As emphasize in Chapter 1, measurements of drug concentrations in th patient can then be used to adjust the dosage regimen t achieve the desired range of concentrations.